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Stable expression and functional characterization of a Na⁺-taurocholate cotransporting green fluorescent protein in human hepatoblastoma HepG2 cells*

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Key words: bile acids and salts, bile formation, cholestasis, luminescent proteins, organic anion transport

Abstract

Sodium-dependent uptake of bile acids from blood is a liver-specific function which is mediated by the Na⁺-taurocholate cotransporting polypeptide (Ntcp). We report the stable expression of a Na⁺-taurocholate cotransporting green fluorescent fusion protein in the human hepatoblastoma cell line HepG2, normally lacking Ntcp expression. Ntcp-EGFP associated green fluorescence colocalized with Ntcp immunofluorescence in the plasma membrane. In transfected HepG2 cells, the fusion protein mediated the sodium-dependent uptake of the bile acid taurocholate (K_m : 24.6 μ mol/l) and of the anionic steroids estrone-3-sulfate and dehydroepiandrosterone sulfate. We conclude that the Ntcp-EGFP fusion protein follows the sorting route of Ntcp, is functionally identical to Ntcp and could be used to monitor protein trafficking in living HepG2 cells.

Abbreviations: Ntcp, Na⁺-taurocholate cotransporting polypeptide of rat liver; EGFP, enhanced green fluorescent protein; DHEAS, dehydroepiandrosterone sulfate; PCR, polymerase chain reaction.

Introduction

The liver extracts a large variety of amphipathic organic substances from sinusoidal blood. Bile acids are taken up into the liver via specific transport proteins localized in the basolateral hepatocyte membrane. The chief transport system for the uptake of conjugated bile acids is the Na⁺-taurocholate cotransporting polypeptide (Ntcp), which has been isolated from human (Hagenbuch et al., 1994), rat (Hagenbuch et al., 1991)

and mouse (Cattori et al., 1999) liver. Functionally, the rat liver Ntcp mediates saturable Na⁺-dependent bile acid uptake with an apparent Michaelis constant (K_m) of 6–34 μ mol/l for taurocholate (Hagenbuch et al., 1991; Meier et al., 1997; Kullak-Ublick et al., 2000). Structurally, Ntcp consists of 362 amino acids, is exclusively expressed in the basolateral membrane of hepatocytes and has an apparent molecular mass of 51 kD (Stieger et al., 1994). Based upon hydrophobicity analysis, Ntcp possesses seven transmembrane domains and the C-terminal end has been localized on the cytoplasmic side of the plasma membrane (Stieger et al., 1994).

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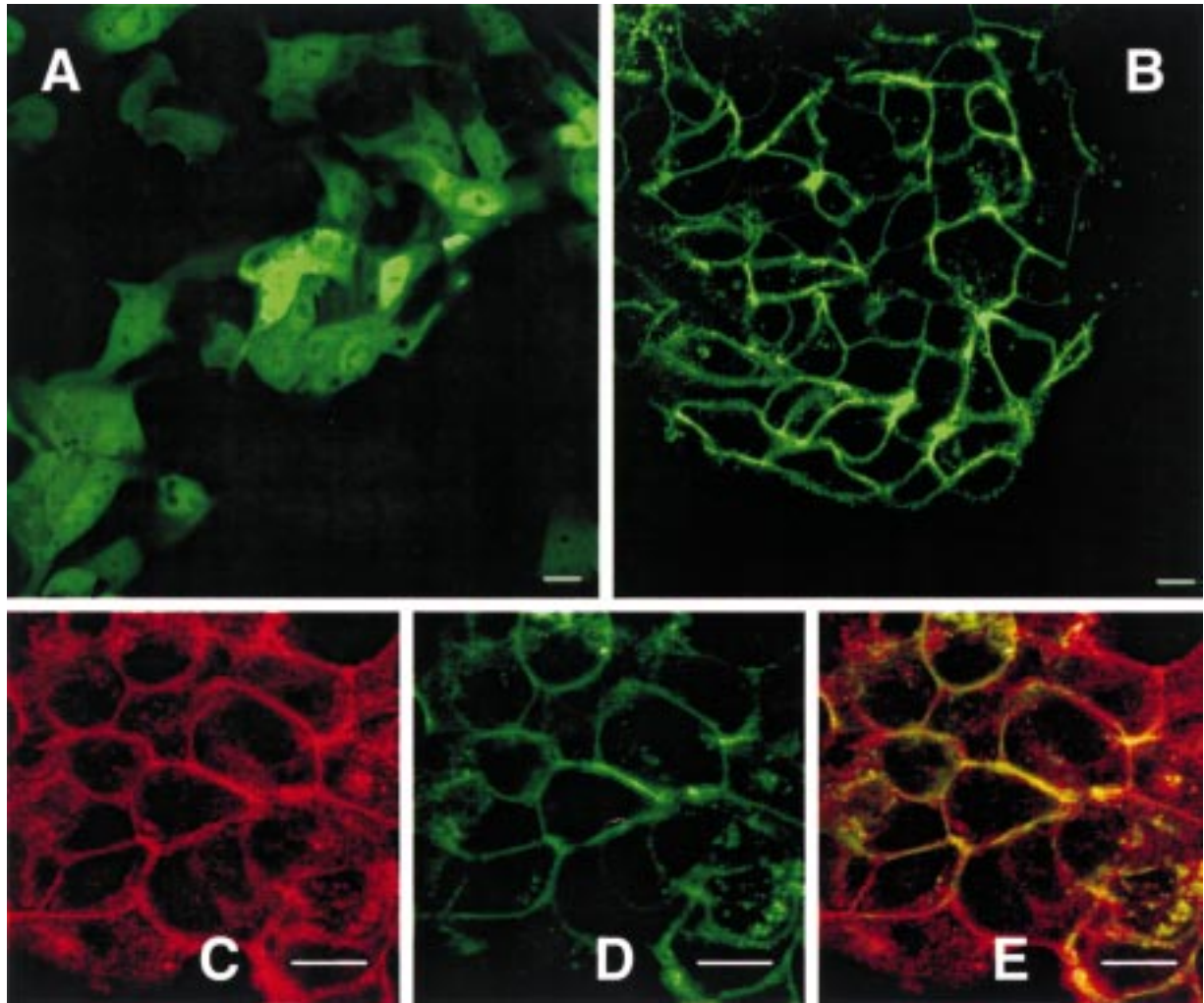


Figure 1. Confocal imaging of HepG2 cells expressing the green fluorescent protein. (A) Transfection of cells with the pEGFP-N1 vector resulted in homogeneous EGFP fluorescence throughout the cytoplasm of the cells, with no particular subcellular localization. (B) In contrast to EGFP fluorescence, the Ntcp-EGFP signal was localized in the plasma membrane, indicating that the fusion protein follows the sorting route of Ntcp. (C) Immunofluorescent staining of Ntcp-EGFP expressing HepG2 cells with the Ntcp antibody (see section Materials and Methods). Imaging of the Ntcp antibody in red (Cy3-fluorescence). (D) EGFP-mediated green fluorescence in the same cells. (E) Overlap of the two fluorescent signals is shown in yellow. The data demonstrate colocalization of Ntcp and the green fluorescent protein, indicating that Ntcp-EGFP is an integral membrane protein. Bars = 10 μ m.

The sodium-dependent uptake of bile acids from blood is a liver-specific function which is lost in numerous models of hepatocellular dedifferentiation. The mRNA coding for the Ntcp protein is rapidly downregulated in primary cultures of rat hepatocytes (Liang et al., 1993). The hepatoblastoma cell line HepG2 is unable to take up bile acids sodium-dependently because Ntcp is not expressed (Boyer et al., 1993; Kullak-Ublick et al., 1996). To be able to use this well characterized liver cell line to study the effects of bile acids – key mediators of liver-specific sig-

nalling mechanisms – on human hepatocytes, it would be essential to create a HepG2 transfectant which expresses Ntcp and is capable of sodium-dependent bile acid uptake. However, stable expression of foreign genes in HepG2 cells is hampered by low transfection efficiencies and loss of the expressed gene after repeated passages. We, therefore, decided to transfect a green fluorescent Ntcp fusion protein. The rationale for using a fluorescent fusion construct rather than normal Ntcp was to visualize the transporter in living cells and thereby to assess the amount of protein

inserted into the plasma membrane. In this study we characterize green fluorescent Ntcp with respect to its *in situ* localization, its functional identity with native Ntcp and its potential usefulness for studying protein trafficking in the unperturbed environment of a living cell.

Materials and methods

Materials

$^3\text{H}(G)$ Taurocholic acid (3.47 Ci/mmol), 6,7- $^3\text{H}(N)$ -estrone-3-sulfate (53.0 Ci/mmol) and 7- $^3\text{H}(N)$ dehydroepiandrosterone sulfate (^3H]DHEAS) (21.1 Ci/mmol) were obtained from DuPont-New England Nuclear, Bad Homburg, Germany. Restriction enzymes were from Boehringer Mannheim, Germany. Unless stated otherwise, all chemicals were purchased from Sigma (Deisenhofen, Germany), Fluka (Heidelberg, Germany) or Merck (Darmstadt, Germany).

Ntcp-EGFP vector construction

The coding region of the rat liver Ntcp from nt 1-1207 of the published cDNA sequence (Hagenbuch et al., 1991) was PCR amplified from the Ntcp plasmid using Pfu DNA polymerase (Stratagene Cloning Systems, Heidelberg, Germany). The upstream primer contained a 5'-flanking *NheI* restriction site, whereas the downstream primer was flanked by a *BamHI* site. The primer sequences used for PCR were as follows: 5'-ATG CTA GCA TCA GCC ACA TTT TGT CCA CAA ACT CTG TCC and 5'-GTG GAT CCC GAT TTG CCA TCT GAC CAG AAT TCA GGC C. The resulting blunt-ended PCR product was cloned into the *SfiI* site of the pCR Script vector (Stratagene Cloning Systems), which was subsequently digested with *NheI* and *BamHI*. The excised Ntcp fragment was ligated in frame into the expression vector pEGFP-N1 (Clontech Corporation, Heidelberg, Germany), which had been predigested with *NheI* and *BamHI*. The plasmid was termed Ntcp-EGFP and contained the full-length Ntcp coding region fused to the N-terminus of the Enhanced Green Fluorescent Protein (EGFP), yielding 2.156 and 2.168 kb cDNAs coding for a 609 amino acid fusion protein. Plasmid DNA was prepared with the Qiagen miniprep system (Qiagen, Hilden, Germany) and was analyzed by restriction digestion and sequencing of the Ntcp insert.

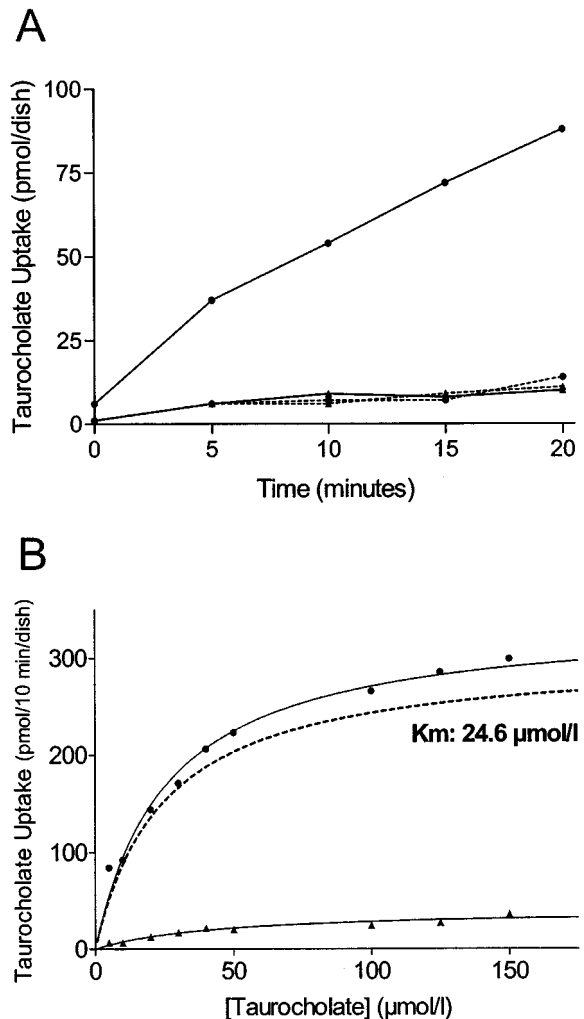


Figure 2. Uptake of taurocholate in transfected HepG2 cells. (A) The uptake of 10 $\mu\text{mol/l}$ [^3H]taurocholate by Ntcp-EGFP expressing (●) and EGFP expressing (▲) cells was measured in a NaCl medium (—) or in a Na^+ -free choline chloride medium (- - -). Uptake of taurocholate was significantly higher in Ntcp-EGFP than in EGFP expressing cells. The Ntcp mediated portion of uptake was strictly sodium-dependent, indicating that Ntcp-EGFP functions as a sodium cotransporter. (B) Kinetics of Ntcp-EGFP mediated taurocholate uptake. Uptake of [^3H]taurocholate by Ntcp-EGFP expressing (●) and EGFP expressing (▲) cells was measured over 10 min in a NaCl medium in the presence of increasing substrate concentrations. Subtraction of uptake values in EGFP expressing cells yielded net Ntcp-EGFP mediated transport (- - -). The apparent K_m was calculated at 24.6 $\mu\text{mol/l}$, which corresponds to the K_m of Ntcp mediated taurocholate uptake in oocytes (see Hagenbuch et al., 1991). The data indicate that Ntcp-EGFP is functionally identical to Ntcp. Results are expressed as the means of four separate uptake measurements.

Culture and stable transfection of HepG2 cells

HepG2 cells (human hepatoblastoma, ATCC HB-8065) were maintained in minimum essential medium

supplemented with 1% nonessential amino acids, 10% fetal calf serum, 1 mmol/l sodium pyruvate and 2 mmol/l glutamine. Cells were transfected with the Ntcp-EGFP or pEGFP-N1 plasmid, using the cationic liposome Tfx-50 (Promega, Heidelberg, Germany) as described (Kullak-Ublick et al., 1997). Following transfection, cells were cultured for 48 h and were subsequently reseeded into culture flasks containing complete culture medium supplemented with 400 mg/l geneticin (G418) (Life Technologies, Eggenstein, Germany). Positive transfectants were identified on the basis of cell-associated fluorescence using a Zeiss axiovert 25 fluorescence microscope (Oberkochen, Germany) and a filter setup for the detection of FITC-fluorescence.

Immunofluorescent staining of Ntcp-EGFP

Untransfected HepG2 cells or HepG2 cells transfected with Ntcp-EGFP or EGFP were grown on uncoated glass coverslips. They were fixed with pure methanol (-20°C , 10 min), washed with PBS and then covered with Triton-X 100 (0.1% in PBS, 10 min). Unspecific binding was blocked with bovine serum albumin (1% in PBS, 1 h). Thereafter the cells were incubated for 2 h in a wet chamber with rabbit anti-rat Ntcp antibody (Stieger et al., 1994) (1:50), or with PBS for control staining. After rinsing and washing, the cells were incubated for 2 h with Cy3-conjugated goat anti-rabbit IgG (1:500) (Jackson Immuno Research Laboratories, West Grove, PA, U.S.A.). After a final washing procedure, the coverslips were mounted on slides with mounting medium (DABCO 2% in glycerol:PBS, 9:1).

Confocal laser scanning microscopy

Living HepG2 cells (on coverslips, placed in a chamber) or immunostained HepG2 cells on slides were mounted on a Leica DM IRB inverted microscope (Leica, Bensheim, Germany). Fluorescent imaging was performed using a Leica TCS-NT confocal laser scanning system with an argon-krypton laser (Leica). Images were acquired from one channel at 488 nm (EGFP fluorescence alone) or from two channels at 488 nm and 568 nm wavelength for immunostained cells. Crosstalk between EGFP- and Cy3-fluorescence was excluded by appropriate adjustments of the excitation light (by use of an acousto-optical tunable filter) and by suiting the voltage of the photo multiplier tubes for the detection of the emitted light. Colocalization of the green (EGFP) and red (Cy3) fluorescent dyes resulted in a yellow signal.

Substrate uptake in EGFP and Ntcp-EGFP expressing HepG2 cells

Uptake of [^3H]taurocholic acid, [^3H]estrone-3-sulfate and [^3H]DHEAS was measured in either a sodium chloride medium or in a sodium-free medium in which NaCl was replaced by choline chloride (Kullak-Ublick et al., 1996). Cells plated in 12-well culture dishes were prewashed with the respective uptake medium and incubated at 37°C in 0.5 ml of the same medium containing the tracer substrate at the indicated concentrations. Uptake was stopped by removing the incubation medium and washing the cells with 3×1 ml ice-cold uptake buffer supplemented with 1 mmol/l of unlabelled substrate to reduce non-specific binding. Cells were subsequently lysed in 1 ml of 1% Triton X-100 and a 200 μl aliquot was removed for protein determination (BCA system, Pierce, Rockford, IL). The cell lysate was dissolved in 10 ml scintillation fluid (Ultima Gold, Canberra Packard, Frankfurt/Main, Germany) and cell-associated radioactivity was counted in a Packard Tri-Carb 2100 liquid scintillation analyzer (Packard Instrument Co., Frankfurt/Main, Germany).

Statistical analysis

Uptake measurements are given as the mean ± 1 standard deviation. The level of significance of taurocholate, estrone-3-sulfate and DHEAS uptake in Ntcp-EGFP vs. EGFP expressing HepG2 cells was determined using the unpaired Student's *t* test (Motulsky, 1995). Statistical significance was assumed at $p < 0.05$.

Results

Expression of Ntcp-EGFP in HepG2 cells

Transfection of HepG2 cells with the Ntcp-EGFP and EGFP plasmids resulted in the detection of green fluorescence in only a small proportion of cells after 48 h. To induce stable expression in the positive transfectants, cells were subjected to G418 selection pressure. After two weeks in culture there was progressive enrichment of fluorescent clones. A comparison of Ntcp-EGFP transfected cells with EGFP transfected control cells showed the same proportion of transfectants for both proteins. After several rounds of subcloning, HepG2 populations with approximately 70% positively expressing cells were finally obtained.

To study the subcellular localization of Ntcp-EGFP, we used confocal laser scanning microscopy.

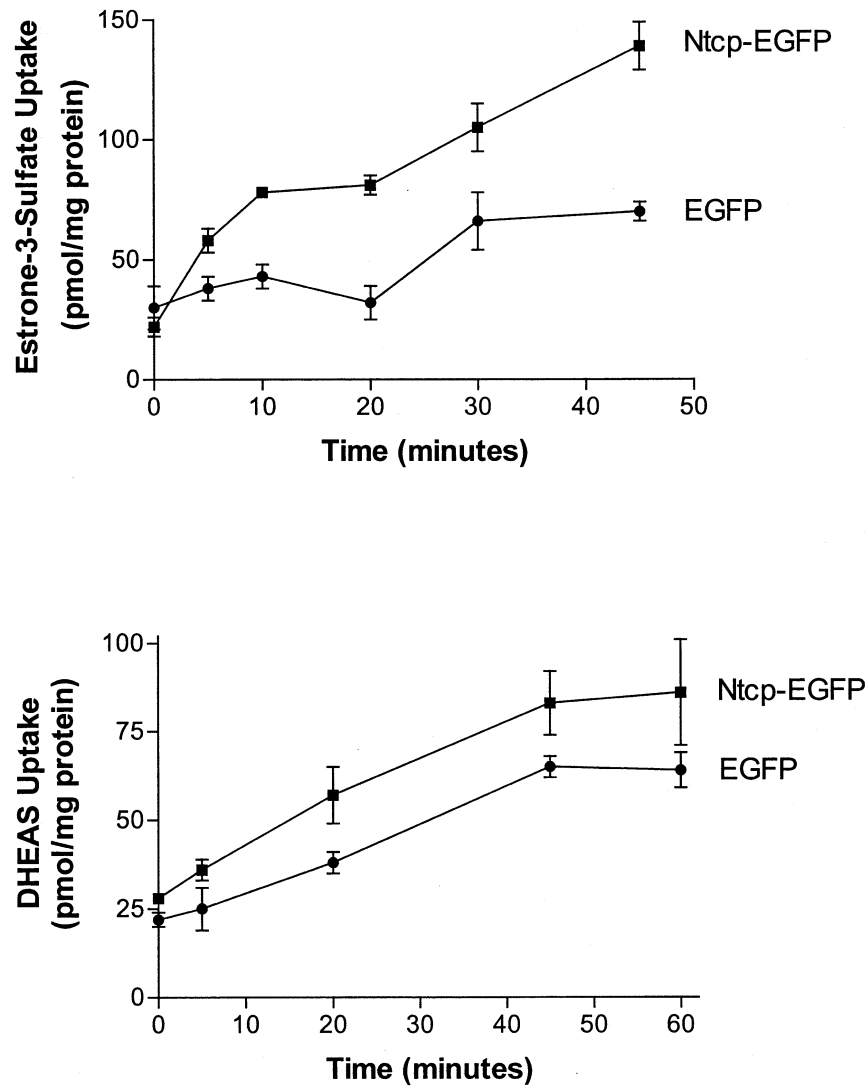


Figure 3. Uptake of [^3H]estrone-3-sulfate ($10\ \mu\text{mol/l}$) and [^3H]DHEAS ($10\ \mu\text{mol/l}$) by EGFP (●) and Ntcp-EGFP (■) expressing HepG2 cells in a NaCl medium. Both substrates were taken up to a significantly higher degree in Ntcp-EGFP cells, indicating that estrone-3-sulfate and DHEAS represent Ntcp substrates. However, endogenous uptake of DHEAS by control cells (EGFP) was higher than net Ntcp mediated uptake. Results are expressed as the means \pm one standard deviation of four separate uptake measurements.

In EGFP expressing HepG2 cells, there was homogeneous EGFP fluorescence throughout the cytoplasm of the cells, with no particular subcellular localization (Figure 1A). In contrast, Ntcp-EGFP fluorescence was localized almost exclusively in the plasma membrane on the basolateral surface (Figure 1B). To confirm the localization of the Ntcp moiety of the fusion construct we performed immunofluorescence using a specific Ntcp antibody (Stieger et al., 1994). The Ntcp signal was localized to the plasma membrane (Figure 1C) and simultaneous imaging of the green fluorescent

protein (Figure 1D) and the Cy3-conjugated Ntcp antibody (Figure 1C) showed complete colocalization of the two proteins (Figure 1E). These results indicated that the Ntcp-EGFP fusion protein is localized on the cell surface and therefore follows the sorting route of Ntcp.

Functional characterization of Ntcp-EGFP mediated taurocholate transport

To investigate whether the fusion protein fulfils the function of Ntcp as a bile acid transporter, two im-

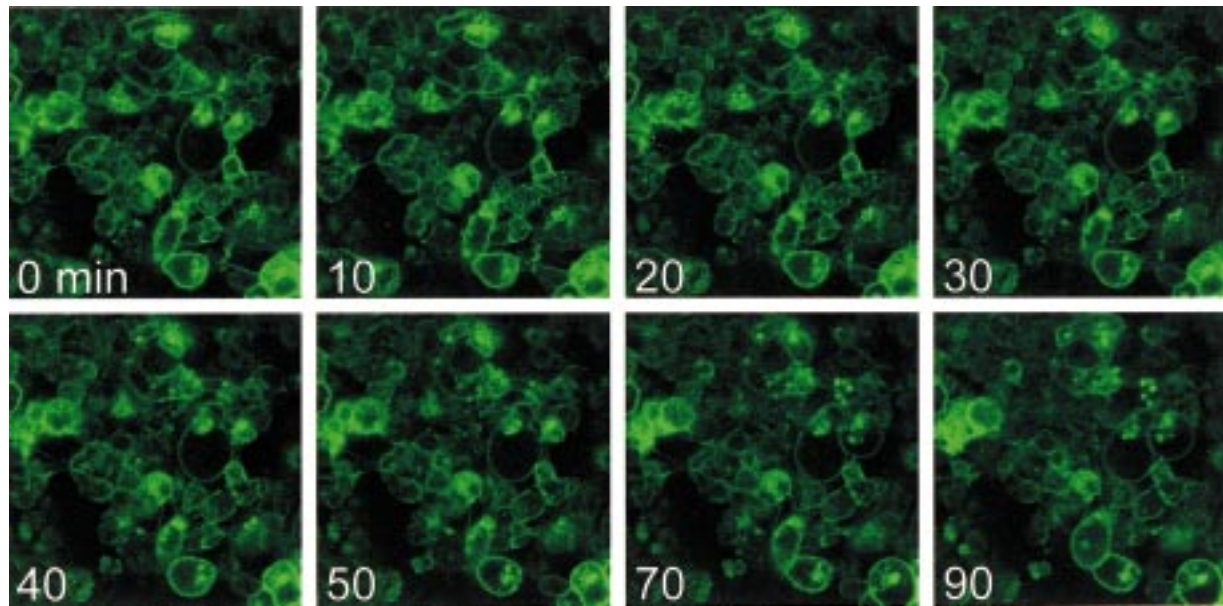


Figure 4. Effect of taurocholate ($10 \mu\text{mol/l}$) on plasma membrane targeting of Ntcp-EGFP. Membrane fluorescence of Ntcp-EGFP expressing HepG2 cells was monitored before (0 min) and at various intervals after (10–90 min) the addition of taurocholate to the medium to study whether membrane insertion of Ntcp is affected by Ntcp substrate. No detectable effect of taurocholate on Ntcp-EGFP membrane fluorescence was observed.

portant features of Ntcp-mediated transport were taken into consideration. First, Ntcp mediates the uptake of bile acids in a strictly sodium-dependent fashion. Second, the substrate specificity of Ntcp is not limited solely to bile acids, but extends to selected organic anions such as estrone-3-sulfate (Meier et al., 1997; Tan et al., 1999). Uptake of the bile salt taurocholate ($10 \mu\text{mol/l}$) by Ntcp-EGFP expressing vs. EGFP expressing HepG2 cells was measured in a sodium chloride medium and in a Na^+ -free choline chloride medium. There was significant sodium-dependent taurocholate uptake by Ntcp-EGFP expressing cells (Figure 2A). In contrast, uptake by EGFP expressing control cells, which lacked Ntcp, was low and sodium-independent. After 20 min, uptake of taurocholate by Ntcp-EGFP expressing cells amounted to $88 \pm 3.7 \text{ pmol}$ in a NaCl medium, compared to $10 \pm 0.7 \text{ pmol}$ in EGFP expressing cells ($p < 0.0001$). In a Na^+ -free choline chloride medium there was no significant difference in uptake between Ntcp-EGFP and EGFP expressing cells (Figure 2A).

We next analyzed the kinetics of Ntcp-EGFP mediated taurocholate transport to compare the affinity of the fusion protein with normal Ntcp. Uptake of taurocholate by Ntcp-EGFP and EGFP expressing cells was measured over 10 min, since separate experiments had shown linear uptake at high ($150 \mu\text{mol/l}$)

and low ($2 \mu\text{mol/l}$) substrate concentrations over this period of time (data not shown). Uptake by Ntcp-EGFP expressing cells was saturable and of high affinity, with an apparent K_m of $24.6 \mu\text{mol/l}$ and a V_{max} of $303 \text{ pmol/10 min/dish}$ after subtraction of Ntcp-independent uptake measured in EGFP expressing cells (Figure 2B). This K_m value is identical to the K_m of Ntcp mediated uptake, which has been calculated at $25 \mu\text{mol/l}$ (Hagenbuch et al., 1991). Uptake of taurocholate by EGFP expressing cells, although quantitatively much lower, was also found to be saturable with an apparent K_m of $44.0 \mu\text{mol/l}$ and a V_{max} of $40.7 \text{ pmol/10 min/dish}$. This K_m value is in the range of the K_m of sodium-independent taurocholate uptake previously reported for normal HepG2 cells (Kullak-Ublick et al., 1996). Collectively, the kinetic data show that Ntcp-EGFP has the same affinity as Ntcp for taurocholate, indicating that the fusion protein retains the functional properties of Ntcp.

Ntcp-EGFP mediated transport of anionic steroids

Based upon transport studies in *X. laevis* oocytes (Meier et al., 1997) and uptake studies in rat hepatocytes (Tan et al., 1999), the anionic steroid estrone-3-sulfate has been shown to be a substrate of Ntcp. To study whether the Ntcp-EGFP fusion pro-

tein is also capable of transporting sulfated steroids, the time course of estrone-3-sulfate and dehydroepiandrosterone sulfate (DHEAS) uptake was measured in Ntcp-EGFP expressing compared to EGFP expressing HepG2 cells in a NaCl medium (Figure 3). For estrone-3-sulfate, uptake after 45 min by Ntcp-EGFP and EGFP cells amounted to 139 ± 10 pmol/mg protein and 70 ± 4 pmol/mg protein, respectively ($p < 0.0001$), indicating additional transport by Ntcp. Nonspecific binding as determined at 0 min was not significantly different between the two cell lines. For DHEAS, uptake by Ntcp-EGFP expressing cells after 45 min (83 ± 9 pmol/dish) was marginally higher than in EGFP cells (65 ± 3 pmol/dish; $p < 0.01$). In a sodium-free choline chloride medium, uptake of estrone-3-sulfate and DHEAS in Ntcp-EGFP expressing cells was not higher than in cells expressing EGFP (data not shown), indicating sodium-dependence of Ntcp-EGFP mediated steroid uptake.

Effect of taurocholate on Ntcp-EGFP membrane localization

To study whether the presence of Ntcp substrate affects the insertion of Ntcp-EGFP into the membrane, membrane fluorescence was analyzed in living cells before and after the addition of taurocholate ($10 \mu\text{mol/l}$) to the medium. As shown in Figure 4, despite moderate changes in cell morphology, no detectable effect upon membrane fluorescence was seen over the first 90 min following the addition of taurocholate. Thus taurocholate appears to affect neither the delivery of Ntcp-EGFP to the plasma membrane nor insertion into the membrane.

Discussion

This study reports the stable expression of a green fluorescent Ntcp fusion protein in the hepatoblastoma cell line HepG2. Cells were transfected with a recombinant fusion construct which contained the full coding region of Ntcp fused to the N-terminus of EGFP. Based on hydrophobicity analysis, Ntcp probably represents a glycoprotein with seven transmembrane domains (Hagenbuch et al., 1991), an extracellular glycosylated N-terminus (Hagenbuch et al., 1996) and an intracellular C-terminus (Stieger et al., 1994). The green fluorescent protein was linked to the intracellular C-terminus of the protein and it thus seems probable that the EGFP moiety was localized

intracellularly. To study the targeting and function of the chimeric protein, we assessed whether Ntcp-EGFP is analogous to the native protein with respect to cell surface localization and preservation of sodium-dependent bile acid transport. Compared to control transfected cells which expressed only EGFP, Ntcp-EGFP was localized in the plasma membrane (Figure 1B). There was colocalization of Ntcp-EGFP associated green fluorescence and Ntcp immunofluorescence (Figure 1E). On a functional level, Ntcp-EGFP mediated sodium-dependent uptake of the known Ntcp substrates taurocholate and estrone-3-sulfate, and to a very minor extent also of dehydroepiandrosterone sulfate, in HepG2 cells (Figures 2 and 3). The kinetics of taurocholate uptake showed high-affinity transport with an apparent K_m of $24.6 \mu\text{mol/l}$ (Figure 2B), which is identical to the K_m of Ntcp mediated taurocholate uptake (Hagenbuch et al., 1991). Collectively, the data indicate that Ntcp-EGFP follows the sorting route of Ntcp and that the fusion protein retains the functional characteristics of Ntcp-mediated transport.

The rationale for transfecting Ntcp-EGFP rather than native Ntcp was to visualize the expressed protein in living cells. In our experience stable expression of foreign cDNAs in HepG2 cells tends to result in low transfection efficiencies and loss of the exogenous gene after repeated passages, underscoring the advantage of direct visualization of a fluorescent chimera for the quantification of gene expression. By subcloning fluorescent cells we obtained a HepG2 population with approximately 70% positive transfectants. This proportion could be enhanced further by fluorescence activated cell sorting. The tagging of peptides with the green fluorescent protein has previously allowed the visualization of intracellular trafficking routes, such as the ER-to-Golgi pathway (Presley et al., 1997). Examples of GFP-tagged proteins which have been visualized in transfected cells include the rat glucocorticoid receptor in mouse cells (Htun et al., 1996), the gamma-aminobutyric acid receptor in *Xenopus* oocytes (Connor et al., 1998) and the multidrug resistance gene product *mdr1b* in polarized hepatoma cells (Sai et al., 1998). The fact that Ntcp-EGFP is targeted correctly to the plasma membrane and is functionally identical with native Ntcp makes the HepG2/Ntcp-EGFP cell line a useful tool for studying intracellular protein localization, in particular the effects of inhibitors of organelle function and of activators of signal transduction pathways on Ntcp transport to the plasma membrane (Dranoff et al., 1999). The addition

of the bile salt taurocholate to the cell medium had no detectable effect upon the intensity of membrane fluorescence (Figure 4).

The finding that Ntcp transports non-bile acid anionic steroids (Figure 3) has previously been reported for estrone-3-sulfate in *Xenopus laevis* oocytes (Meier et al., 1997). The overall uptake of estrone-3-sulfate by hepatocytes occurs via sodium-dependent and sodium-independent mechanisms (Tan et al., 1999). The results of this study indicate that DHEAS also represents a weak Ntcp substrate. Because plasma DHEAS concentrations can reach 10 $\mu\text{mol/l}$ (Baulieu, 1996), uptake experiments were performed at that concentration. The liver is a major site of metabolism of DHEAS into sex steroids and active metabolites are released from the hepatocyte back into the circulation (Baulieu, 1996). The major uptake systems for DHEAS in the liver are the multispecific organic anion transporting polypeptides (Kullak-Ublick et al., 1998; Abe et al., 1999). The high degree of estrone-3-sulfate and DHEAS uptake in control HepG2 cells expressing only EGFP could be attributable to the endogenous expression of organic anion transporting polypeptide in HepG2 cells (Kullak-Ublick et al., 1996).

Conclusions

We have generated a HepG2 cell line which stably expresses a green fluorescent Na^+ -taurocholate cotransporting fusion protein. Ntcp-EGFP is an integral membrane protein and therefore follows the sorting route of Ntcp. In transfected cells it mediates the uptake of taurocholate with the same affinity as native Ntcp and also transports the anionic steroids estrone-3-sulfate and to a minor degree DHEAS. The fluorescent Ntcp-EGFP chimera should be a useful tool for studying the regulation of intracellular protein trafficking in response to physiological regulators of organelle function.

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